

DESCRIPTIONMULTI-SUBTYPE FIV VACCINES

5 The subject invention was made with government support under a research project supported by National Institutes of Health Grant No. NIH AI30904. The government has certain rights in this invention.

Cross-Reference to Related Applications

10 This application is a continuation of U.S. application Serial No. 10/116,196, filed April 3, 2002, now U.S. Patent No. 6,605,282, which is continuation of U.S. application Serial No. 09/512,746, filed October 1, 1997, now U.S. Patent No. 6,447,993, which is a file wrapper continuation of U.S. application Serial No. 08/519,386, filed August 25, 1995, now abandoned.

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Background of the Invention

20 Domestic cats are subject to infection by several retroviruses, including feline leukemia virus (FeLV), feline sarcoma virus (FeSV), endogenous type C oncoronavirus (RD-114), and feline syncytia-forming virus (FeSFV). Of these, FeLV is the most significant pathogen, causing diverse symptoms including lymphoreticular and myeloid neoplasms, anemias, immune-mediated disorders, and an immunodeficiency syndrome that is similar to human acquired immune deficiency syndrome (AIDS). Recently, a particular replication-defective FeLV mutant, designated FeLV-AIDS, has been more particularly associated with immunosuppressive properties.

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 The discovery of feline T-lymphotropic lentivirus (now designated as feline immunodeficiency virus, FIV) was first reported in Pedersen *et al.* (1987). Characteristics of FIV have been reported in Yamamoto *et al.* (1988a); Yamamoto *et al.* (1988b); and Ackley *et al.* (1990). Seroepidemiologic data have shown that infection by FIV is indigenous

to domestic and wild felines throughout the world. A wide variety of symptoms are associated with infection by FIV, including abortion, alopecia, anemia, conjunctivitis, chronic rhinitis, enteritis, gingivitis, hematochezia, neurologic abnormalities, periodontitis, and seborrheic dermatitis. The immunologic hallmark of domestic cats infected with FIV is a chronic and progressive depletion of feline CD4⁺ peripheral blood lymphocytes, a reduction in the CD4:CD8 cell ratio and, in some cases, an increase in CD8-bearing lymphocytes. Based on molecular, biochemical and immunopathologic characteristics, FIV infection of cats is now considered to be a better feline AIDS model than FeLV-FAIDS.

Cloning and sequence analysis of FIV has been reported in Olmsted *et al.* (1989a); Olmsted *et al.* (1989b); and Talbott *et al.* (1989). Hosie and Jarret (1990) described the serological response of cats infected with FIV. FIV virus subtypes can be classified according to immunotype based on the level of cross-neutralizing antibodies elicited by each strain (Murphy and Kingsbury, 1990). Recently, viruses have been classified into subtypes according to genotype based on nucleotide sequence homology. Although HIV and FIV subtyping is based on genotype (Sodora *et al.*, 1994; Rigby *et al.*, 1993; and Louwagie *et al.*, 1993), little is known about the correlation between the genotype and immunotype of subtypes. FIV viral isolates are currently classified into four FIV subtypes: A, B, C and D. (Kakinuma *et al.*, 1995). Infectious isolates and infectious molecular clones have been described for all FIV subtypes except for subtype C (Sodora *et al.*, 1994). Subtype C FIV has only been identified from cellular DNA of cats from Canada (Sodora *et al.*, 1994; Rigby *et al.*, 1993; Kakinuma *et al.*, 1995).

A major difficulty in developing an FIV vaccine has been in identifying a vaccine approach that is effective against a broad range of FIV strains including field isolates from different subtypes or clades. Vaccine prophylaxis for FIV has been attained against 25 homologous and slightly heterologous strains using a single-strain vaccine, but not against challenge with moderate to greatly heterologous strains (Johnson *et al.*, 1994; Yamamoto *et al.*, 1993). Thus, there remains a need for a vaccine that protects across multiple FIV subtypes.

Brief Summary of the Invention

The subject invention concerns a vaccine that elicits a broad range of protective immunity against FIV infections in a host animal. Specifically, the subject invention concerns a multi-subtype FIV vaccine that is prepared using cell-free viral isolates from different FIV subtypes, or a combination of cell lines each infected with a different prototype FIV virus from a different subtype. Cats vaccinated with the FIV vaccines of the subject invention develop humoral and cellular immune responses to homologous and heterologous FIV strains.

The subject invention also concerns novel feline cell lines that are susceptible to infection by multiple FIV subtypes. The cell lines of the subject invention are useful for propagating and producing multiple FIV subtypes, as well as for use in FIV vaccines according to the methods of the subject invention. In addition, the cell lines can also be used in place of feline peripheral blood mononuclear cells (PBMC) in FIV viral neutralization assays of feline antisera.

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Brief Description of the Drawings

Figure 1 shows the reverse transcriptase (RT) levels of FIV_{Bang} and FIV_{Shi} produced after infecting FeT-1C and FeT-J cell lines with these FIV strains.

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Figure 2 shows the immunoreaction of anti-FIV antibodies from dual-subtype vaccinated cats with FIV proteins as detected by immunoblot. The number over each blot represent the number of vaccinations received by the animal when the sera was tested.

Figure 3 shows the immunoreaction of anti-FIV antibodies from triple-subtype vaccinated cats with FIV proteins as detected by immunoblot. The number over each blot represent the number of vaccinations received by the animal when the sera was tested.

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Figure 4 shows the immunoreactivity of anti-FIV antibodies from triple-subtype vaccinated cats with FIV SU-V3-2 peptide as detected by ELISA.

Figure 5 shows the immunoreactivity of anti-FIV antibodies from triple-subtype vaccinated cats with FIV TM-C1 peptide as detected by ELISA.

Figure 6 shows cross-neutralizing antibody titers of sera from cats infected with either FIV_{Pet} (A_P), FIV_{Dix} (A_D), FIV_{UK8} (A_U), FIV_{Bang} (B_B), FIV_{Aom1} (B_A), and FIV_{Shi} (D_S). Sera at pre-infection (column 1), 6 months post-infection (column 2), and 12 months post-infection (column 3) were tested against subtype A FIV_{Pet}, subtype B FIV_{Bang}, and subtype D FIV_{Shi} in the FeT-1C-cell line. At least three cats per each strain were tested and results show VN titer from a representative cat from each strain. Similar results were obtained using primary PBMC for VN assay.

Brief Description of the Sequences

SEQ ID NO. 1 is an amino acid sequence of an FIV surface envelope peptide designated as SV-V3-2.

SEQ ID NO. 2 is an amino acid sequence of an FIV transmembrane peptide designated as TM-C1.

SEQ ID NO. 3 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 4 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 5 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 6 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 7 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 8 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 9 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 10 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 11 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 12 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 13 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 14 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 15 is a nucleotide sequence of an FIV PCR primer.

SEQ ID NO. 16 is a nucleotide sequence of an FIV PCR primer.

Detailed Disclosure of the Invention

The subject invention concerns novel methods and vaccine compositions useful for inducing protective immunity to FIV infection in a susceptible host animal. The vaccine compositions described herein, when administered to a host animal, induce protective humoral and cellular immune responses against infection by homologous and heterologous strains of FIV. The vaccine compositions may comprise either cell-free FIV viral isolates or FIV-infected cell lines. In a preferred embodiment, the vaccine composition of the subject invention comprises FIV strains from two different FIV subtypes. Preferably, the vaccine composition comprises three FIV strains, each strain from a different FIV subtype. More preferably, at least one FIV strain from each of FIV subtype A, subtype B and subtype D is included in the vaccine composition.

In a specific embodiment, the vaccine composition comprised FIV_{Pet-} and FIV_{Shi-} infected cell lines. In another embodiment, the vaccine composition comprised FIV_{Pet-}, FIV_{Bang-}, and FIV_{Shi-}infected cell lines. The use of other FIV strains representative of all or a portion of FIV subtypes is specifically contemplated by the subject invention. For example, FIV_{Dix} or FIV_{UK8} could be included in the vaccine compositions in addition to or in place of FIV_{Pet} for purposes of providing an FIV subtype A prototype virus. Similar additions or substitutions with other FIV strains could be made for FIV subtype B and D prototype viruses.

The multi-subtype FIV vaccines specifically described herein were tested for immunogenicity and efficacy in cats. Specific pathogen free (SPF) cats vaccinated with the subject vaccine compositions were monitored for humoral and cellular immune responses before and after challenge with homologous and heterologous FIV strains. Humoral responses were monitored by measuring viral neutralizing (VN) antibody activity and cellular responses were monitored by measuring cytotoxic T lymphocyte (CTL) activity. Sera and immunocytes from vaccinated cats were tested *in vitro* for VN and CTL activities, respectively, against homologous and heterologous FIV strains, and demonstrated that the vaccines can elicit broad-range protection from FIV infection. According to the teachings of

the subject invention, by combining prototype virus isolates from different subtypes, or by combining individual cells infected with prototype virus of different subtypes, an effective multi-subtype FIV vaccine can be produced.

All FIV strains, in addition to those specifically exemplified herein, are contemplated for use with the subject invention. A number of FIV isolates have been described in the literature and are known to those skilled in the art. FIV_{pet} has been described in U.S. Patent No. 5,037,753. Other FIV isolates which have been described can be readily isolated from infected cats by persons of ordinary skill in the art using standard techniques. Methods for isolating and culturing FIV are described in U.S. Patent Nos. 5,037,753 and 5,118,602, which are herein incorporated by reference.

The novel cell lines exemplified herein can be used in the vaccine methods and compositions of the present invention. Other cells or cell lines that are susceptible to infection by FIV strains, including peripheral blood mononuclear cells, are also contemplated for use with the present invention.

Natural, recombinant or synthetic polypeptides of FIV viral proteins can also be used as vaccine compositions according to the subject methods. In a preferred embodiment, FIV polypeptides derived from multiple FIV subtypes are combined in a vaccine composition and are used to vaccinate a host animal. For example, polypeptides based on the FIV envelope glycoprotein from at least two prototype FIV strains from different subtypes can be combined in the vaccine. The polypeptides may be homologous to one strain or may comprise "hybrid" or "chimeric" polypeptides whose amino acid sequence is derived from joining or linking polypeptides from at least two distinct FIV subtypes. Procedures for preparing FIV polypeptides are well known in the art.

The present invention also concerns novel feline T-cell lines that are susceptible to infection by FIV. Both interleukin-2 (IL-2) dependent and independent cells are specifically exemplified. The cell lines designated as FeT-1C and FeT-J are described herein. The FeT-1C cell line is IL-2 dependent, whereas the FeT-J cell line is IL-2 independent. The cell lines of the subject invention are useful for providing a vehicle for FIV immunization of cats, as

well as for propagating and producing FIV viral strains *in vitro*. Both the IL-2-dependent FeT-1C and IL-2-independent FeT-J uninfected cell lines were tested over 20 times for reverse transcriptase (RT) activity in culture fluids and for FIV proviral sequence by PCR and were confirmed negative for FIV. FeT-J cell line was highly infectable with all of the FIV 5 strains tested, including FIV_{Shi}, FIV_{Dix}, FIV_{UK8}, FIV_{Pet} and FIV_{Bang} but was more difficult to directly infect with FIV_{Shi}.

10 The subject invention further concerns cellular products produced by the cell lines of the present invention. The cellular products can be isolated and detected using procedures known to the skilled artisan. Antibodies to the cell lines can also be produced using known methods and are contemplated by the subject invention.

15 The FIV uninfected cell lines designated as FeT-1C (ATCC Accession No. CRL 11968) and as FeT-J (ATCC Accession No. CRL 11967) were both deposited with the American Type Culture Collection, Rockville, Maryland on August 24, 1995. FIV_{Bang}- (ATCC Accession No. 11975) and FIV_{Shi}- (ATCC Accession No. 11976) infected cell lines were deposited with the American Type Culture Collection on August 25, 1995.

20 The subject cultures have been deposited under conditions that assure that access to the culture will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. 122. The deposit will be available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

25 Further, the subject culture deposit will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., it will be stored with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the culture. The depositor

acknowledges the duty to replace the deposit should the depository be unable to furnish a sample when requested, due to the condition of the deposit. All restrictions on the availability to the public of the subject culture deposit will be irrevocably removed upon the granting of a patent disclosing it.

5 According to the methods of the subject invention, the FIV vaccine compositions described herein are administered to susceptible hosts, typically domestic cats, in an effective amount and manner to induce protective immunity against subsequent challenge or infection of the host by FIV. The vaccines are typically administered parenterally, by injection, for example, either subcutaneously, intraperitoneally, or intramuscularly. Other suitable modes 10 of administration include oral or nasal administration. Usually, the vaccines are administered to a host at least two times, with an interval of one or more weeks between each administration. However, other regimens for the initial and booster administrations of the vaccine are contemplated, and may depend on the judgment of the practitioner and the particular host animal being treated.

15 The vaccine compositions of the subject invention can be prepared by procedures well known in the art. For example, the vaccines are typically prepared as injectables, *e.g.*, liquid solutions or suspensions. The vaccines are administered in a manner that is compatible with dosage formulation, and in such amount as will be therapeutically effective and immunogenic in the recipient. The optimal dosages and administration patterns for a particular vaccine 20 formulation can be readily determined by a person skilled in the art.

25 As described herein, the vaccine compositions of the subject invention may comprise cell-free whole FIV virus, or portions of the virus, FIV proteins and polypeptides, as well as FIV-infected cell lines, or a combination of cell-free virus and infected cell lines. Vaccine compositions comprising FIV-infected cell lines may comprise multiple cell lines, each infected with a different FIV subtype. Virus and cells in a vaccine formulation may be inactivated or attenuated using methods known in the art. The amount of cell-free whole FIV virus in a vaccine dose will usually be in the range from about 0.1 mg to about 5 mg, and more usually being from about 0.2 mg to about 2 mg. The dosage for vaccine formulations

comprising FIV-infected cell lines will usually contain from about 10^6 to about 10^8 cells per dose, and more usually from about 5×10^6 to about 7.5×10^7 cells per dose.

Virus or cells were typically combined with an adjuvant just prior to administration. Adjuvants used in the vaccine formulations typically were either threonyl muramyl dipeptide (MDP) (Byars *et al.*, 1987) or a combination of Freund's complete and incomplete adjuvants. A variety of other adjuvants suitable for use with the methods and vaccines of the subject invention, such as alum, are well known in the art and are contemplated for use with the subject invention.

The subject invention further concerns a novel method for assaying for virus neutralizing (VN) antibodies in a sample using the uninfected cell lines of the present invention. Unlike PBMC which expire after a limited number of passages and do not propagate as readily as FeT-1C or FeT-J cells, the FeT-1C and FeT-J cells are an established cell line and can be readily cryopreserved for future use. Results obtained from VN assays using FeT-1C cells are more highly reproducible than VN assays using PBMC because PBMC from different SPF cats have individual variability in cell growth rate and FIV infectability. Further, PBMC for VN assays have to be obtained from SPF cats which require germ-free housing and maintenance in order to eliminate possible *in vivo* infection which may affect an *in vitro* VN assay using PBMC. Thus, a feline cell line such as FeT-1C which can be readily infected with FIV of different subtypes can be advantageously substituted for PBMC in VN assays.

The following abbreviations of FIV strains are used herein:

<u>Strain (subtype)</u>	<u>Abbreviation</u>
Petaluma (A)	FIV _{Pet}
Dixon (A)	FIV _{Dix}
UK8 (A)	FIV _{UK8}
Bangston (B)	FIV _{Bang}
Aomori-1 (B)	FIV _{Aom1}
Aomori-2 (B)	FIV _{Aom2}
Shizuoka (D)	FIV _{Shi}

Materials and Methods

Cell cultures. All suspension cell lines were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS). 10mM HEPES (N-2-hydroxyethylpiperazine-n'-2-ethane sulfonic acid), 2 mM L-glutamine, 50 μ g/ml gentamicin and 5×10^{-5} M 2-mercaptoethanol. IL-2-dependent cells were supplemented with 100 U/ml of recombinant human IL-2 (Cetus Corporation, Emeryville, Calif.). The suspension cells were passaged at a cell concentration of $0.5-4 \times 10^6$ cells/ml and recultured in fresh culture media twice a week. All monolayer cells were passaged twice a week at an initial cell concentration of 2×10^6 cells/ml. The tissue culture fluids (TCF) from FIV-infected cells were harvested twice a week, spun at 3000 rpm for 1 hour to remove residual cells, and stored at -20° C, or at -70° C for those TCF scheduled to be used immediately upon testing. FIV-susceptible cells (1×10^6 cells/ml) were infected with FIV having a reverse transcriptase (RT) activity of about 30,000 cpm/ml.

FIV purification. Tissue culture fluids from FIV-infected cell lines were individually centrifuged at 2000 to 3000 rpm for 1 hr to remove cells. Virus in the TCF was pelleted by ultracentrifugation at 16,000 rpm for 2 hours, and purified by ultracentrifugation first on a 10/50% (w/v) discontinuous sucrose gradient and then on a 10/50% continuous sucrose gradient (Pederson *et al.*, 1987; Yamamoto *et al.*, 1988). Each of the viral isolates was inactivated with 1.25% sterile paraformaldehyde (0.22 μ m sterile filtered) for 18 hr and subsequently extensively dialyzed against sterile PBS. The inactivated viruses were diluted to a concentration of 500 μ g/ml with sterile PBS and 250 μ g/0.5 ml of each strain was placed in sterile microfuge tube and stored at -70° C. The inactivated FIV strains were thawed at room temperature and 250 μ g of inactivated virus in 0.5 ml sterile PBS was combined with 0.5 ml of adjuvant just prior to immunization. FIV-infected cell lines were separately inactivated with 1.25% sterile paraformaldehyde for 18 hr, washed 3 times with sterile PBS, resuspended in fresh sterile PBS at concentration of about 5.0×10^7 cells/ml in sterile tubes and stored at 4° C. Typically, about 2.5×10^7 inactivated infected cells in 0.5 ml sterile PBS

were combined with 0.5 ml of adjuvant just prior to immunization. 250 µg/0.5 ml of threonyl muramyl dipeptide (MDP MF75.2 adjuvant; Chiron Corporation, Emeryville, CA) was used as an adjuvant.

CTL assay. Peripheral blood mononuclear cells (PBMC) were stimulated with 5 Concanavalin A (Con A) for 3 days prior to infection with FIV for 10 days (Song *et al.*, 1992). These cells served as target cells for the CTL assay. CTL activity was generated by co-culturing Con A-stimulated PBMC with autologous UV- and radiation inactivated FIV-infected PBMC for 5 days. These cells served as the stimulated effector cells. On the assay 10 day, target cells were labeled with 50µCi of Na⁵¹CrO₄ for 1 to 3 hours, washed 3 times, and then a fixed number of labeled target cells (5x10⁴ cells/well) were added to microtiter plates. Effector cells were added in triplicate at various effector/target cell ratios (i.e., 100:1, 50:1, and 10:1). Plates were centrifuged for 1 minute at 400 rpm and incubated at 37° C for 4 hours. Control ⁵¹Cr-labeled target cells were lysed with detergent to obtain maximal release values. Supernatants from the test sample wells were collected and radiation was quantified 15 using a gamma counter. Spontaneous release was determined by incubating ⁵¹Cr-labeled target cells in the absence of effector cells. Percentage of specific cytotoxicity was calculated as:

$$\text{% cytotoxicity} = (100) \frac{(\text{mean cpm test release} - \text{mean cpm spontaneous release})}{(\text{mean cpm maximum release} - \text{mean cpm spontaneous release})}$$

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Immunoblot and enzyme linked immunosorbent assays (ELISA). Sucrose gradient purified virus was used as substrate for an immunoblot assay as described in Yamamoto *et al.*, 1993. FIV_{pet} from tissue culture fluid of infected cells was clarified by low speed centrifugation (2000 rpm for 45 min), concentrated by ultracentrifugation (16,000 rpm for 25 2 hr), and purified by ultracentrifugation on a 10/50% (w/v) continuous sucrose gradient. The virus purified by this procedure was used as the substrate for the immunoblot assay.

A modification of an immunoblot technique previously described was used (Yamamoto *et al.*, 1991a). Virus blot strips were prepared by solubilizing virus in 0.1% SDS, followed by electrophoresis on 10% SDS-polyacrylamide gel and electrophoretic transfer

onto nitrocellulose membrane. Serum samples from vaccinated cats were diluted to 1:50 in Buffer 3 (0.15 M sodium chloride, 0.001 M editic acid, 0.05 M TRIS base, 0.05% Tween 20, and 0.1% bovine serum albumin) and incubated with virus blot strips in separate wells of immunoblot plate for 18 hrs at 37°C. The blot strips were washed individually with wash 5 solution (0.15 M NaCl and 0.05% Tween 20 in deionized H₂O), incubated with biotinylated anti-cat IgG (Vector Laboratories, Burlingame, CA) for 1 hr at 37°C, and washed three times with wash solution. The strips were then incubated individually with horseradish peroxidase conjugated Streptavidin (Vector Laboratories) for 30 min. After extensive washing, each strip was incubated with a fresh substrate solution (0.05% diaminobenzidine, 400 µg/ml 10 NiCl₂, and 0.01% H₂O₂ in 0.1 M Tris buffer, pH 7.4) at room temperature. The reaction was stopped with excess distilled H₂O upon establishment of visible bands, and the strips were blot dried. The molecular weights of the bands on the immunoblots were then determined by 15 comparing them with the migration distance of the molecular weights standards on a strip previously stained with amido black. Positive and negative control serum were included in each immunoblot analysis as internal controls for diagnostic evaluation.

The viral antigen-specific ELISA has been previously described (Yamamoto *et al.*, 1991a; Yamamoto *et al.*, 1993). Sucrose gradient purified FIV_{pet} and surface envelope (SU) and transmembrane (TM) peptides of both conserved (C) and variable (V) regions of FIV_{pet} were coated on 96 well Immunolon plates (Dynatech Laboratories, Inc., Chantilly, VA) at 20 250 ng/well with bicarbonate buffer (pH 9.6) for 12 to 18 hours at 37°C and were used as substrates for ELISA. The amino acid sequence of the SU-V3-2 peptide is: Gly Ser Trp Phe Arg Ala Ile Ser Ser Trp Lys Gln Arg Asn Arg Trp Glu Trp Arg Pro Asp Phe (SEQ ID NO. 1); and the amino acid sequence of the TM-C1 peptide is: Gln Glu Leu Gly Cys Asn Gln Asn Gln Phe Phe Cys Lys Ile (SEQ ID NO. 2). The synthetic peptides were synthesized on a Biosearch 9500 peptide synthesizer (Biosearch, San Rafael, CA) using Fmoc peptide synthesis chemistry (Magazine *et al.*, 1988). Purity of the synthesized peptides was determined by the presence of a single peak on a reversed-phase high-performance liquid 25

chromatography and confirmed by amino acid sequence analysis performed on the peak sample.

The peptide coated plates were washed once with Buffer 3 immediately prior to use. The serum samples were diluted at 1:200 in Buffer 3 and incubated in the FIV antigen coated wells for 1 hr at 37° C, then washed 6 times. The wells were washed with wash solution, incubated with biotinylated anti-cat IgG (Vector Laboratories, Burlingame, CA) for 1 hr at 37°C, washed 6-times, and incubated with horseradish peroxidase conjugated Streptavidin (Vector Laboratories) for 1 hr at 37°C. The wells were then washed 6 times with wash solution and incubated with ELISA substrate solution (0.005% tetramethylbenzidine and 10 0.015% H₂O₂ in 0.96-% citrate solution) at room temperature. The reaction was stopped with 0.1 M hydrofluoric acid upon establishment of visible reaction color in the sequentially diluted standards consisting of known FIV-positive cat serum. Light absorption was measured with a BioRad ELISA reader (Bio-Rad Laboratories, Hercules, CA) at optical density of 414 nm.

15 Polymerase Chain Reaction (PCR). The proviral DNA levels of infected cells were monitored by differential PCR, which was recently developed to distinguish multiple FIV strains from the same or different subtypes (Okada *et al.*, 1994). As a means of increasing the sensitivity of PCR, the nested PCR primer sets shown in Table 1 were used. PCR was performed in a two stage reaction, first with a pair of outer primers (common for all FIV 20 strains) under conditions as described in Okada *et al.*, 1994. In the second PCR stage, 1/25 of the first stage product was amplified using the inner primers (specific for each FIV strain). Using nested PCR, cells infected with FIV_{Pet}, FIV_{UK8}, FIV_{Bang}, FIV_{Aom1}, FIV_{Aom2} and FIV_{Shi} can be distinguished from each other.

Table 1. Primer sets for differential PCR.

Subtype	Strain	Primer (orientation)	Sequence	Position*
<u>Outer Primer Sets</u>				
All	NA	common (+) common (-)	GAAATGTATAATTGCTGG (SEQ ID NO. 3) GAATTGATTGGATTACATCC (SEQ ID NO. 4)	1570-1589 2112-2092
<u>Inner Primer Sets</u>				
A	Petaluma	Pet (+)	TAGTAGTTATAGTGGTACTA (SEQ ID NO. 5)	1659-1678
		Pet (-)	TCTTTAAGGCTTCAGTCACCT (SEQ ID NO. 6)	1984-1964
UK-8	UK8 (+) UK8 (-)		GTACAAATAGTAGTAGTACAA (SEQ ID NO. 7) TCTTTAAGGCTTCAGTCACCT (SEQ ID NO. 8)	1646-1666 1984-1964
		Bang (+) Bang (-)	GGGACTACTAGCAATGGAATA (SEQ ID NO. 9) AGTGCCCTCAGTTATTTATCC (SEQ ID NO. 10)	1654-1674 1979-1959
Aomori-1	Aol (+) Aol (-)		TGGGACTGATGATAAGTAAAC (SEQ ID NO. 11) AGTGCCCTCAGTTATTTATCC (SEQ ID NO. 12)	1654-1674 1979-1959
Aomori-2	Ao2 (+) Ao2 (-)		TGGGACTGATAATAGTGAAC (SEQ ID NO. 13) AGTGCCCTCAGTTATTTATCC (SEQ ID NO. 14)	1654-1674 1979-1959
D	Shizuoka	Shi (+) Shi (-)	TCATCATTTCACACATGTC (SEQ ID NO. 15) AATGCTTCAGTTATTTGATC (SEQ ID NO. 16)	1663-1681 1979-1960

* Nucleotide positions correspond to those of Petaluma sequence and the numbers represent the position from the start of *env* sequence.

The approximate amount of proviral DNA per cell was determined by semi-quantitative PCR, in which varying dilutions of DNA extracted from a known number of cells are made. For example, if 10^5 cells are used for DNA extraction, then a 10^{-5} dilution of the DNA preparation will approximately correspond to the DNA present in a single cell. PCR was performed on these varying DNA dilutions and the final dilution that gave a positive PCR result is considered the end-point dilution. The number of cells corresponding to the end-point dilution is used to determine the percentage of cells infected with virus in a given cell preparation according to the following formula:

$$\% \text{ infected cells} = \frac{1}{Z} \times 100$$

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where Z=the number of cells corresponding to the end-point dilution.

Reverse transcriptase (RT) assay. The presence of RNA-dependent DNA polymerase (RT) was assayed in cell culture supernatants essentially as described by Rey *et al.* The RT assay for detecting FIV used poly(rA)-oligo(dT₁₂₋₁₈) as an exogenous template primer, four different deoxyribonucleotide triphosphates, 20 mM KCl with Mg⁺⁺ as divalent cation and 5 μ Ci [³H]-labeled thymidine triphosphate (TTP) per sample. Five μ Ci [³H]TTP gave an average total count of 1,200,000 cpm using scintillation fluid mixture (1 part xylene to 9 part Research Products International biodegradable counting scintillant) on a Beckman LS250 scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). As a result, RT values for samples tested will be below 1,200,000 cpm/ml.

Viral neutralization assay. A strategy for developing strain- and subtype-specific VN assays has been described (Okada *et al.*, 1994). Serial dilutions of heat-inactivated sera were incubated with 100 TCID₅₀ of each FIV strain for 45 minutes at 37° C in a 24-well plate prior to addition of feline peripheral blood mononuclear cells (PBMC) (4×10^5 cells/ml) or FIV-susceptible FeT-1C cells (2×10^5 cells/ml). After 3 days of culturing, the cells were washed once with Hank's balanced salt solution to remove residual virus from the culture and then the cells were resuspended in fresh culture media (RPMI-1640 containing 10% heat-inactivated fetal calf serum, 10 mM HEPES buffer, 50 μ g/ml gentamicin, 5×10^{-5} M 2-mercaptoethanol, and 100 Units/ml human recombinant IL-2). Virus infection of cells was monitored by Mg⁺⁺-

dependent RT assays of the culture fluids harvested on 9, 12, 15, and 18 days of culture. Sera were considered positive for VN antibodies when RT activity was $\leq 25\%$ of infected control cultures consisting of SPF serum.

5 Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – FIV-infected cell lines.

10 A novel interleukin-2 (IL-2) dependent feline T-cell line, designated as FeT-1C, which is a mother line of an IL-2-dependent FeT-1M clone, was used to establish individual cell lines chronically infected with either FIV_{Pet}, FIV_{Dix}, FIV_{UK8}, FIV_{Bang}, FIV_{Aom2}, or FIV_{Shi}. The FeT-1M clone (also referred to as FIV-Fet1M) has been described in U.S. Patent No. 5,275,813, which is herein incorporated by reference, and was used to produce an IL-2-independent cell 15 line, FL-4 (also described in U.S. Patent No. 5,275,813), that chronically produces FIV_{Pet}. The FeT-1C cell line is highly infectable with different isolates from FIV subtypes A, B, and D. Long-term passaging of the FeT-1C cell line decreases its infectability, especially to FIV subtype D; therefore, the passage number should be less than about 35 passages for optimal 20 FIV infection rates or for its use in VN assays. Semi-quantitative PCR and viral core antigen analyses indicated that all the cell lines exposed to FIV were significantly infected with individual FIV strains.

25 An IL-2 independent feline cell line susceptible to FIV infection has also been developed from FeT-1C cells. This cell line, designated as FeT-J, can be infected with FIV by co-culture using FIV infected media or cells. For example, an FIV_{Bang}-infected FeT-1C cell line was co-cultured in the absence of IL-2 with uninfected FeT-J cells to establish an IL-2-independent FIV_{Bang}-infected FeT-J cell line (designated as Bang/FeT-J). In the co-culture method of infection, Bang/FeT-1C cells were combined with uninfected FeT-J cells at a ratio of from about 2:1 to about 10:1 (infected:uninfected). The cell mix was cultured in media in

the absence of IL-2 for several days and the FeT-1C cells were allowed to die off. The remaining cells consisted of FIV_{Bang}-infected FeT-J cells. Thus, FIV-infected FeT-1C cells can be used to infect FeT-J cells and establish IL-2-independent FeT-J cell lines infected with different FIV subtypes. The co-cultivation method with FIV infected FeT-1C cells resulted 5 in IL-2-independent FeT-J cell lines producing moderate to high levels of different FIV subtypes.

The FeT-1C cell line was also infected with FIV_{Shi} and extensively passaged to produce an IL-2-dependent cell line designated as Shi/FeT-1C. The Shi/FeT-1C cell line was later co-cultured with FeT-J in the absence of IL-2 and the resulting IL-2-independent FIV_{Shi}-10 infected cell line was designated as Shi/FeT-J. The IL-2-independent Shi/FeT-J cell line produces higher levels of FIV_{Shi} than IL-2-dependent Shi/FeT-1C cell line (Figure 1).

The development of a FeT-J cell line infected with FIV_{Bang} was also performed without the use of the FeT-1C cell line. The FeT-J cell line was directly infected with cell-free FIV_{Bang} inoculum and extensively passaged without IL-2. The resulting IL-2-independent FIV_{Bang} 15 producer cell line was designated Bang/FeT-J. The Bang/FeT-J cell line produced higher levels of FIV_{Bang} than the IL-2-dependent Bang/FeT-1C cell line which was developed by infecting FeT-1C cell line with FIV_{Bang} (Figure 1).

Example 2 – Multi-subtype FIV vaccines.

20 FIV-infected cells were removed from supernatants by centrifugation, inactivated, and used as vaccine. Similarly, whole FIV virus were pelleted from infected cell-free supernatant by ultracentrifugation and inactivated. Both infected cells and virus were inactivated by treatment with 1.25% paraformaldehyde for 24 hours at 5°C, followed by extensive washing or dialysis against PBS, respectively. This method efficiently inactivates FIV without loss of 25 immunogenicity. FIV immunogens produced according to the subject method are highly effective for inducing protective immunity (Yamamoto *et al.*, 1993; Yamamoto *et al.*, 1991a; Yamamoto *et al.*, 1991b). It is contemplated that attenuated viral isolates could also be used in the vaccine compositions of the subject invention.

Although an FIV_{Shi}-infected FeT-1C cell line was superinfected with the FIV_{Pet} strain to produce a single cell line infected with multiple subtypes of FIV (i.e., a multi-subtype A/D FeT-1C cell line), within two months of co-infection the FIV_{Shi} proviral levels decreased from 50% to less than 5% whereas FIV_{Pet} proviral levels concomitantly increased to about 50%.
5 Thus, the maintenance of a single cell line infected with multiple subtypes of FIV for use as an FIV vaccine is not the preferred embodiment of the subject invention.

Consequently, in one embodiment of the subject invention, vaccine compositions were developed from two individual cell lines, each line being infected with a different FIV subtype. In a specific embodiment, the dual-subtype FIV vaccine composition comprised a combination 10 of an FIV subtype A-infected cell line (Pet/FL-4) with an FIV subtype D-infected cell line (Shi/FeT-1C). The A-subtype and D-subtype infected cell lines were inactivated as described, combined in equal cell numbers (2.5×10^7 cells each in 250 μ g of MDP) and used to immunize cats. Three SPF cats were vaccinated with inactivated Pet/FL-4 cells and four other cats were vaccinated with inactivated Shi/FeT-1C cells (2.5×10^7 cells/dose). After a series of four 15 vaccinations, the dual-subtype (Pet/FL-4 and Shi/FeT-1C) vaccine induced anti-FIV antibodies, including significant VN antibody titers, to both FIV strains tested (Figure 2 and Table 2, Trial I). Four dual-subtype (Pet/FL-4 and Shi/FeT-1C) vaccinated cats were challenged with FIV_{Bang} (50 CID₅₀). All three Pet/FL-4 vaccinated and two of the Shi/FeT-1C vaccinated cats were challenged with 50 CID₅₀ of FIV_{Bang}. The two remaining Shi/FeT-1C 20 vaccinated cats were challenged with 50 CID₅₀ of FIV_{Shi}.

All dual-subtype vaccinated cats were negative for FIV_{Bang} by virus isolation and PCR of PBMC at 6 weeks post-infection (pi), whereas all sham immunized cats were positive for either FIV_{Bang} or FIV_{Shi} by virus isolation and PCR at 6 weeks post-infection (Table 2, trial I). In contrast, one cat each from Pet/FL-4 vaccinated and Shi/FeT-1C vaccinated groups 25 which was challenged with FIV_{Bang} was positive for FIV_{Bang}. As expected, all cats vaccinated with FIV_{Shi} and subsequently challenged with FIV_{Shi} were negative for FIV_{Shi} at 6 weeks post-infection. Thus, the dual-subtype vaccine specifically exemplified prevented or delayed

infection against homologous FIV_{Shi} challenge as well as against heterologous FIV_{Bang} challenge.

The dual-subtype vaccinated cats (Pet/FL-4 cells and Shi/FeT-1C cells) developed FIV antibodies specific for the viral core protein p25 (also call FIV p28) after the second immunization (Figure 2). Higher antibody titers to other viral antigens were demonstrated after the third to fourth immunization. VN antibodies to FIV_{Pet} developed after the second immunization, whereas VN antibodies to FIV_{Shi} developed after the fourth immunization (Table 4). CTL responses to FIV_{Pet} and FIV_{Shi} were detected as early as the third immunization in all cats tested (Table 3) and stronger CTL responses to both strains were developed after the fourth immunization. Further, two of the three cats tested developed CTL responses to FIV_{Bang} after the fourth immunization. Results indicate that after 4 vaccinations, the dual-subtype vaccine induced strong CTL responses to FIV_{Pet} and FIV_{Shi} (Table 3) and high FIV antibodies, including VN antibody titers, to both FIV strains (Table 4).

The cats immunized with inactivated Shi/FeT-1C cells developed FIV antibodies specific for the viral core protein p25 after the second immunization and antibodies to other viral antigens after the third immunization (Figure 2). VN antibodies to FIV_{Shi} in these cats developed after the fourth immunization, whereas VN antibodies to FIV_{Pet} were not detected over the course of the immunizations. Both of the Shi/FeT-1C vaccinated cats developed CTL responses to FIV_{Shi} only after the fourth immunization but did not develop CTL responses to FIV_{Pet}, even after the fourth immunization (Table 3).

Cats immunized with inactivated Pet/FL-4 cells developed antibodies to p25 after the second immunization (Figure 2) and to other viral antigens, including VN antibodies to FIV_{Pet}, after the second to third immunization (Table 4). The only CTL responses detected in cats immunized with Pet/FL-4 cells were to FIV_{Pet}. Overall, the dual subtype FIV vaccine induced more rapid and higher VN antibody titers and CTL responses to both FIV strains than the single-subtype vaccine. Sham immunized SPF cats did not develop viral antibodies, VN antibodies, or anti-FIV CTL responses.

Table 2. Protection of cats with multi-subtype FIV vaccine

Vaccine type	No. of Cats	FIV challenge strain (CID ₅₀) ¹		Average VN Antibodies at Day 0 pi against ²	Virus Isolation & PCR	Protection Rate (%)
		Pet	Shi			
Dual-subtype Vaccine Trial I (A+D):						
Pet/FL-4 cell + Shi/FeT-1C cell	4	FIV _{Bang} (50 CID ₅₀)	1000	550 <10	All Negative	4/4 (100% at 6 wk pi)
Pet/FL-4 cell ³	3	FIV _{Bang} (50 CID ₅₀)	1000	<10	2/3 Negative	2/3 (67% at 6 wk pi)
Shi/FeT-1C cell	2	FIV _{Bang} (50 CID ₅₀)	<10	75 <10	1/2 Negative	1/2 (50% at 6 wk pi)
Shi/FeT-1C cell	2	FIV _{Shi} (50 CID ₅₀)	<10	30 <10	All Negative	2/2 (100% at 6 wk pi)
sham	4	FIV _{Bang} (50 CID ₅₀)	<10	<10	All Positive	0/4 (0% at 6 wk pi)
sham	2	FIV _{Shi} (50 CID ₅₀)	<10	<10	All Positive	0/2 (0% at 6 wk pi)
Triple-subtype Vaccine Trial II (A+B+D):⁴						
Pet/FL-4 cell+Bang/FeT-J cell +Shi/FeT-1C cell ⁵	3	FIV _{UK3}	1000	370	1000	
Bang/FeT-J cell	2	FIV _{UK3}	<10	<10	1000	
Bang/FeT-J cell	2	FIV _{Bang}	<10	<10	100	
Sham Uninfected FeT-J	2	FIV _{UK3}	<10	<10	<10	
Uninfected FeT-J	2	FIV _{UK3}	<10	<10	<10	
Sham Adjuvant only	1	FIV _{UK3}	<10	<10	<10	
Uninfected FeT-J	1	FIV _{Bang}	<10	<10	<10	
Sham Adjuvant only	2	FIV _{Bang}	<10	<10	<10	

¹ All FIV challenge inocula were produced *in vitro* by infecting primary PBMC from SPF cats. All aliquoted inocula were stored in -70° C and thawed at room temperature just prior to use.

² pi = post FIV infection.

³ ND = not done.

⁴ VN results are after third vaccination.

⁵ Fourth vaccination will be performed with inactivated Shi/FeT-J cells instead of inactivated Shi/FeT-1C cells.

Table 3 – CTL responses from dual-subtype vaccinated cats.

Cat#	Vaccine Type	CTL Target	CHROMIUM RELEASE (% LYSIS)					
			3rd Vaccination		4th Vaccination			
			Effector:Target Ratio	10:1 50:1 100:1	Effector:Target Ratio	10:1 50:1 100:1	Effector:Target Ratio	10:1 50:1 100:1
K55	Pet + Shi	Pet	0	9	20	17	25	33
		Bang	ND	ND	ND	0	0	14
		Shi	0	9	8	7	11	17
3L4	Pet + Shi	Pet	0	11	19	0	11	19
		Bang	ND	ND	ND	0	0	0
		Shi	0	9	13	0	9	17
N55	Pet + Shi	Pet	ND	ND	ND	0	11	17
		Bang	ND	ND	ND	0	0	7
		Shi	ND	ND	ND	0	9	15
M55	Shi	Pet	0	0	0	0	0	0
		Bang	ND	ND	ND	0	0	0
		Shi	0	0	0	0	7	15
007	Shi	Pet	0	0	0	0	0	0
		Bang	ND	ND	ND	0	0	0
		Shi	0	0	0	0	0	8
2H5D	Pet	Pet	0	7	15	6	15	25
		Bang	ND	ND	ND	0	0	0
		Shi	0	0	0	0	0	0
3G1	Pet	Pet	0	10	14	6	13	19
		Bang	ND	ND	ND	0	0	0
		Shi	0	0	0	0	0	0
H7P	Sham	Pet	0	0	0	0	0	0
		Bang	ND	ND	ND	0	0	0
		Shi	0	0	0	0	0	0

Table 4. Virus neutralization (VN) titers from dual-subtype vaccinated cats.

Cat No.	FIV Vaccine	Pre-Vaccination			Post 2nd Vaccination			Post 4th Vaccination		
		Pet	Bang	Shi	Pet	Bang	Shi	Pet	Bang	Shi
K55	Pet + Shi	>10	>10	>10	100	<10	<10	1000	<10	100
	Pet + Shi	>10	>10	>10	100	<10	<10	1000	<10	1000
	Pet + Shi	>10	>10	>10	10	<10	<10	1000	<10	1000
	Pet + Shi	>10	>10	>10	10	<10	<10	1000	<10	100
M55	Shi	>10	>10	>10	>10	>10	>10	<10	<10	50
	Shi	>10	>10	>10	>10	>10	>10	<10	<10	100
	Shi	>10	>10	>10	>10	>10	>10	<10	<10	10
	Shi	>10	>10	>10	>10	>10	>10	<10	<10	50
3G1	Pet	>10	>10	>10	<10	<10	<10	1000	<10	<10
	Pet	>10	>10	>10	10	<10	<10	1000	<10	<10
	Pet	>10	>10	>10	100	<10	<10	1000	<10	<10
8C2	Sham	>10	>10	>10	>10	>10	>10	>10	>10	>10
	Sham	>10	>10	>10	>10	>10	>10	>10	>10	>10
	Sham	>10	>10	>10	>10	>10	>10	>10	>10	>10
	Sham	<10	<10	<10	<10	<10	<10	<10	<10	<10
8G8	Sham	<10	<10	<10	<10	<10	<10	<10	<10	<10
	Sham	<10	<10	<10	<10	<10	<10	<10	<10	<10
	RF5	<10	<10	<10	<10	<10	<10	<10	<10	<10
	RF5	<10	<10	<10	<10	<10	<10	<10	<10	<10

In a preferred embodiment, the vaccine composition of the subject invention comprises a triple-subtype FIV vaccine prepared from three cell lines, each cell line having been infected with a viral strain from a different FIV subtype (A or B or D). Three specific pathogen free cats were immunized with a triple-subtype (FIV_{Pet} + FIV_{Bang} + FIV_{Shi}) vaccine. 5 Other cats were immunized with single-subtype FIV_{Bang} vaccines to evaluate the immunogenicity of macrophage-tropic FIV_{Bang} as a component of the vaccine. The VN antibody titer results indicate that both triple-subtype (FIV_{Pet}+FIV_{Bang}+FIV_{Shi}) and single-subtype FIV_{Bang} vaccines elicited high antiviral antibody titers even after the second immunization (Table 2, trial II and Table 5). Thus, both lymphotropic and macrophage-tropic 10 FIV can be used as components of the vaccine compositions of the present invention.

The three SPF cats immunized with a combination of inactivated Pet/FL-4, inactivated Bang/FeT-J, and inactivated Shi/FeT-1C cells (2.5×10^7 cells each in 250 μ g total of MDP) developed FIV antibodies specific for the viral core protein p25 and to other viral antigens, including FIV SU and TM envelope protein, after the second immunization (Figures 3, 4, 5). 15 VN antibodies to FIV_{Pet}, FIV_{Bang} and FIV_{Shi} developed in the majority of cats soon after the second immunization and in all cats by the third immunization (Table 5). In addition, one cat had VN antibodies that cross reacted to FIV_{UK8} after third immunization. Four SPF cats immunized only with inactivated Bang/FeT-J cells developed FIV antibodies specific for the viral core protein p25 and other viral antigens after the second immunization (Figure 3). VN 20 antibodies to FIV_{Bang} in these cats developed after the second immunization (Table 5), whereas VN antibodies to FIV_{Pet} and FIV_{UK8} were not detected over the course of the immunizations. CTL responses of cats immunized three times with the triple-subtype FIV vaccine (Pet/FL-4, Bang/FeT-J and Shi/FeT-1C cells) to FIV A, B and D subtype target cells are shown in Table 6. CTL responses to all three FIV subtypes tested were detected. Thus, 25 the triple-subtype vaccine induced a broad CTL response and more rapid and higher VN and SU-envelope antibody titers than the single-subtype vaccine. Neither uninfected FeT-J nor Sham immunized SPF cats developed viral antibodies or VN antibodies.

Table 5 – Virus neutralization (VN) titers from triple-subtype vaccinated cats.

CAT#	FIV VACCINE	Pre-Vaccination			Post 2nd Vaccination			Post 3rd Vaccination					
		Pet	Bang	Shi	UK8	Pet	Bang	Shi	UK8	Pet	Bang	Shi	UK8
J55	Pet+Bang+Shi	<10	<10	<10	<10	1000	1000	<10	<10	1000	1000	10	<10
QY1	Pet+Bang+Shi	<10	<10	<10	<10	100	1000	100	<10	1000	1000	<10	<10
TAS	Pet+Bang+Shi	<10	<10	<10	<10	<10	1000	10	<10	100	1000	100	100
BEA	Bang	<10	<10	<10	<10	<10	100	<10	<10	<10	1000	<10	<10
QU8	Bang	<10	<10	<10	<10	<10	10	<10	<10	<10	100	<10	<10
RD2	Bang	<10	<10	<10	<10	<10	100	<10	<10	<10	100	<10	<10
3G4	Uninfected FeT-J	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
3G5	Uninfected FeT-J	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
3G6	Sham	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
3G7	Sham	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

The VN titers are the average titers of two separate VN assays.

Table 6. CTL responses of triple-subtype vaccinated cats after 3rd immunization

Cat No.	Target FIV	E:T Ratio	CTL Activity (% chromium release)
QY1	FIV _{Pet}	100	44%
		50	21%
		10	4%
QY1	FIV _{Bang}	100	13%
		50	6%
		10	1%
5	QY1	100	23%
		50	8%
		10	2%
Tas	FIV _{Bang}	100	8%
		50	3%
		10	1%
Tas	FIV _{Shi}	100	3%
		50	1%
		10	0.3%
J55	FIV _{UK8}	100	10%
		50	2%
		10	1%

10

Example 3 – VN antibodies to FIV subtypes.

An assay for VN antibodies to FIV was also developed using the FeT-1C cells of the subject invention. Serum from FIV_{Pet}-infected cats and SPF cats vaccinated with inactivated Pet/FL-4 cells or inactivated FIV_{Pet} virus were tested for VN antibody titer using either FeT-1C cells or PBMC according to the VN assay method described herein. Sera from two SPF cats which were unvaccinated and FIV uninfected were used as control sera. Sera from vaccinated and FIV infected cats had a high VN antibody titer of 1000 or greater, whereas sera from unvaccinated SPF cats had no detectable VN antibody titer. The FeT-1C-based VN assay gives VN antibody titer results comparable to those obtained using primary PBMC

from cats (Table 6). This finding demonstrates that VN antibody titers in a VN assay using FeT-1C cells correlates with those results obtained with a VN assay using PBMC. Therefore, FeT-1C cells can be advantageously used in place of PBMC in the standard VN assay for FIV since FeT-1C cells can be infected with all the FIV subtypes and can be readily propagated in tissue culture.

Table 7. VN titers assayed on FeT-1C and PBMC

Serum source	VN titers	
	FeT-1C	PBMC
Vaccinated ¹	5000	5000
Vaccinated ¹	>1000	>1000
Infected ²	1000	1000
Infected ²	>1000	>1000
Uninfected cell immunized ³	<10	<10
Uninfected cell immunized ³	<10	<10

¹ - Sera from inactivated Pet/FL-4 cell vaccinated cats

² - Sera from FIV_{pet} infected cats

³ - Sera from inactivated uninfected FeT-J immunized cats

Example 4 – Immunotyping FIV strains

In vitro studies were performed using FeT-1C cells to assess if FIV subtype reflected FIV immunotype. Immunotyping is important for understanding the role of VN antibodies in vaccine protection. Antisera from cats infected with FIV subtype A strains (FIV_{pet}, FIV_{Dix}, FIV_{UK8}), subtype B (FIV_{Bang}, FIV_{Aom1}), and subtype D (FIV_{Shi}) were tested for the ability to neutralize these strains *in vitro* using FeT-1C cells in the VN assay (Figure 6). All of the test antisera had neutralizing activity against the corresponding homologous FIV strain. FIV_{pet},

a subtype A strain, was significantly cross-neutralized by antisera from cats infected with FIV_{Dix}. FIV_{Pet} differs from FIV_{Dix} by approximately 9% at surface envelope glycoprotein (Env) regions. Anti-sera from cats infected with FIV subtype A strains cross-neutralized subtype B FIV_{Bang} but did not neutralize subtype D FIV_{Shi}. Antisera from cats infected with subtype B and D strains only cross-neutralized other FIV strains within the homologous subtype. Further, antisera from cats infected with FIV_{UK8} neutralized FIV_{Bang} but did not neutralize FIV strains within subtype A. Although FIV_{UK8} is classified as subtype A (Sodora *et al.*, 1994; Rigby *et al.*, 1993; Kakinuma *et al.*, 1995), these results suggest that antisera to FIV_{UK8} recognizes subtype B strains, but does not recognize subtype A strains, and may explain why inactivated FIV_{Pet} vaccines were ineffective against FIV_{UK8} and FIV_{Shi} (Johnson *et al.*, 1994). Thus, a loose correlation exists between genotype and immunotype. Although genotypic analyses allow for FIV strain classification, cross-neutralization antibody studies reflect the immunogenicity of FIV strains, which is an important parameter in broad-range humoral protection elicited by vaccines.

15

Example 5 – FIV cell tropism.

The cell tropism of the FIV strains obtained from infected FeT-1C and infected FeT-J cell lines were compared to those FIV strains obtained from primary PBMC (Table 8). Two FIV isolates, FIV_{UK8} and FIV_{Bang}, are both equally lymphotropic and macrophage-tropic, whereas FIV_{Shi} is highly lymphotropic. FIV_{Pet} was more lymphotropic than macrophage-tropic and its cell tropism was not significantly affected by its cell source. The macrophage-tropism of FIV_{Bang} was not affected by the cell source of the virus. Since the cell tropism of the FIV strains from infected FeT-1C cell line is comparable to those produced from primary PBMC the virus grown in FeT-1C cells can be used as inoculum for VN assays and also as an *in vivo* inoculum for studies to evaluate therapeutic and prophylactic approaches.

Table 8. Cell tropism of FIV Isolates.

		TCID _{50^a}					
		FIV (Subtype)	FIV Source	FeT-1C	PBMC	Alveolar Macrophage	Primary Microglia
5	Petaluma (A)	PBMC	10 ⁴	10 ⁴	10 ²	ND	
	Petaluma (A)	FeT-1C ^b	10 ⁴	10 ⁴	10 ¹	ND	
	Petaluma (A)	FL-4 ⁴	10 ⁴	10 ⁴	10 ¹	ND	
	Dixon (A)	FeT-1C	10 ⁴	10 ³	10 ¹	ND	
	UK8 (A)	PBMC	10 ²	10 ³	10 ³	ND	
	UK8 (A)	FeT-1C	10 ³	10 ³	10 ³	ND	
10	Bangston (B)	PBMC	10 ³	10 ³	10 ³	10 ²	
	Bangston (B)	FeT-1C ^b	10 ³	10 ³	10 ³	10 ²	
	Bangston (B)	FeT-J ^b	10 ³	10 ³	10 ³	10 ²	
	Shizuoka (D)	PBMC	10 ²	10 ³	<1	ND	
	Shizuoka (D)	FeT-1C ^b	10 ³	10 ³	<1	ND	
	Shizuoka (D)	FeT-J ^b	10 ³	10 ³	ND	ND	

^a - All virus inocula were adjusted to 120,000 cpm/ml of RT activity before titration on 5x10⁵ cells/ml of feline T cells (FeT-1C) or primary feline cells and the results represents the highest titer of the virus harvested over 21 days of culturing.

^b - Same cells as the infected-cell vaccines.

20

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

25